

Phytochemical analysis, antioxidant and cytotoxic activities of *Dryopteris ramosa*.

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Abstract

Objective: The aim of present study was to evaluate phytochemical, cytotoxic and antioxidant potential of dichloromethane and methanol extracts of *Dryopteris ramosa* (Hope) C. Chr. belongs to family Dryopteridaceae. The plant is traditionally used as anthelmintic, diuretic, antibacterial, astringent and as tonic for various stomach and intestinal problems.

Method: The phytochemical potential, total phenol, flavonoid and alkaloid contents of *Dryopteris ramosa* were determined for the first time. Plant was subjected to Brine shrimp lethality and DPPH inhibition assay was done. Identification and quantification of various phenolic content by was done HPLC.

Results: Phytochemical analysis revealed that flavonoids, terpenoids, cardiac glycosides, alkaloids and saponins were present in the plant while tannins were absent. The results depicted high *in vitro* cytotoxic effect by dichloromethane extract with a LD50 0.6903 µl/ml as standardized with Etoposide with LD50 of 7.4625 µl/ml. The methanol extract showed the strong DPPH scavenging activity with 72.76 ± 1.52% inhibition as compared to the dichloromethane extract with 55.37 ± 3.055% inhibition. The high phenol content was found in methanol extract having 199.2 ± 4.50(mg of GA/g of extract). HPLC analysis showed the presence of various phenol compounds (Gallic acid, quercetin, caffeic acid, vanillic acid, cinnamic acid etc.) with their relevant concentration, in the plant. The dichloromethane extract exhibited high flavonoid content 73.02 ± 1.00 (mg of RE/g of extract). Alkaloidal content was highest in dichloromethane extract with 29.73 ± 4.04 (mg of AE/g of extract).

Keywords: Antioxidant, Cytotoxic, *Dryopteris ramosa*, HPLC, Phytochemical analysis.

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Introduction

Oxidative stress caused by free radicals can cause serious damage to the important cellular macromolecules like DNA and protein [1]. These toxic effects of free radicals have drawn the researcher's attention to the significance of antioxidants in the prevention and treatment of various ailments [2]. Many studies have been done on the natural sources to produce natural antioxidants with lower cytotoxic profiles [3].

Cancer, a genetic disease, is mainly caused due to genetic instability involving variations in tumor suppressor genes and in oncogenes which causes expression of abnormal proteins engaged in the stimulation of cell proliferation and its survival [4]. Previous studies have proved that free radicals have the key role in the development of cancer [5].

Secondary metabolites, like phenols, alkaloids, flavonoids and tannins, widely distributed in plants, possess multiple biological effects including free radical scavenging activities, anti-inflammatory, antioxidant and anti-carcinogenic effects [6]. Various studies have been done on herbal plants, fruits and vegetables which indicated the presence of antioxidants in the

form of phenolic compounds, flavonoids, proanthocyanidins and tannins [7].

Contrariwise, to the best of our understanding, various medicinal plants used as vernacular medicine still require scientific attention and *D. ramosa* is one of them. Plant is traditionally known as "Pakha". *D. ramosa* is used as diuretic, astringent and febrifuge [8]. Plant is also used as tonic for various stomach and intestinal problems [9]. Young leaves are collected in March-May as a vegetable and are used as spinach against gastric ulcer, constipation and as aphrodisiac [10]. Rhizome has anti-bacterial and anthelmintic potential [11]. In the present research dichloromethane and methanol extracts of *D. ramosa* were evaluated for their phytochemical potential, cytotoxic and antibacterial capacities.

Method

Plant collection and extraction

The plant *D. ramosa* was collected from place Nathia gali, District Abbottabad, Khyber Pakhtunkhwa province, Pakistan. Plant was identified by Assistant Professor Dr. Zafarullah

Zafar, Institute of Pure Applied Biology, Bahauddin Zakariya University, Multan. In order to obtain effective extracts plant was dried in shade for about 15 days. Dried plant was ground and weighed. Extraction of plant done was by the process of maceration. The weighed amount of ground plant was kept in extraction bottle and then pre-determined volume of dichloromethane was added to it. For the purpose of maximum extraction bottle was placed on ultrasonic bath. After 24 hours, filtration was carried out. Same process was done thrice with dichloromethane. Further, marc of plant was extracted with methanol in the same manner. The dichloromethane and methanol extracts were prepared using rotary evaporator. The dichloromethane (100.2 g) and methanol (120.9 g) extracts were collected in separate sample bottles and designated with codes as DRD and DRM respectively.

Preliminary phytochemical analysis

Chemical tests for identification of secondary metabolites:

The dry powder of whole plant *D. ramosa* was evaluated for the presence of secondary metabolites (like alkaloids, saponins, cardiac glycosides, etc.) according to the standard protocol methods. In these tests formation of turbidity or development of color is generally involved. An authentic specimen is always used to compare the obtained color. While in case of precipitation reaction turbidity formation is compared with the control. Control is always without drug merely containing reagent. These tests can be applied to the extracts as well as isolated constituents [12].

Determination of total phenol content: Total phenolic content was determined using Folin–Ciocalteu colorimetric method. Gallic acid was used as the standard and the results were expressed in terms of equivalent (GAE) per gram of sample. Various concentrations of gallic acid (0.01-0.1 mg/ml) were prepared using methanol as solvent. Fractions of 0.5 ml of the sample and the standard were taken. Two ml of Folin–Ciocalteu reagent was added to these fractions and the reaction was initiated by the addition of (7.5% w/v) sodium carbonate solution. The tubes were then covered with silver foils and were allowed to incubate for about thirty minutes at room temperature. Absorbance was measured at 765 nm while using methanol as blank. All the samples were processed in three repetitions. The total phenolic content of the extract was determined by the help of standard curve of Gallic Acid. Folin–Ciocalteu is a sensitive reagent that reduces compounds including polyphenols, hence producing a blue color upon reaction. This blue color was measured [13].

Determination of total flavonoid content: Aluminum chloride colorimetric assay was used to determine the flavonoid content of the plant extract in this study. Fractions of 0.5 ml of the sample and the standard, Rutin, (0.01-1.0 mg/ml) of were taken. Two ml of distilled water and 0.15 ml of sodium nitrite (5% NaNO₂, w/v) solution was added to the fractions and then mixed. To this, 10% solution of AlCl₃ w/v, was added after six minutes. The prepared solution was allowed to stand for more six minutes and then 2 ml of (4% NaOH w/v) sodium hydroxide solution was added to it. Dist. water was added

immediately to make the final volume 5 ml. This mixture was allowed to stand for 15 min. Absorbance of prepared mixtures was taken at 510 nm. Calibration curve of Rutin was used to determine total flavonoid content and then TFC was expressed in terms of mg Rutin equivalent per gram of sample. Same procedure was performed thrice [14].

Determination of total alkaloid content: 1 mg of plant extract was taken and dissolved in Dimethyl Sulfoxide (DMSO). 1.0 ml of 2 N HCl was added to it and was then filtered. After filtration, filtrate was transferred to separating funnel. 5 ml of bromocresol green solution and 5 ml of phosphate buffer, was added to the separating funnel. This mixture was vigorously shaken by adding 1,2,3 and 4 ml chloroform. Later, it was collected in 10 ml volumetric flask and was diluted with chloroform to make the volume. Reference standard solutions of atropine (20, 40, 60, 80 and 100 µg/ml) were prepared likewise. The absorbance of test and standard solutions was measured at 470 nm in UV-Spectrophotometer and the total alkaloid content was expressed as mg of AE/g of extract [15].

Identification and quantification of phenol contents by HPLC: Samples (10 µl) were analyzed by HPLC (Shimadzu Model 10A) pump equipped with a UV-Visible detector, 280 nm using a Shim-Pack CLC-ODS (C-18) column, 25 cm × 4.6 cm × 5 µm. Solvents used were A water-AA (94:6, pH=2.27) and B acetonitrile 100% at a flow rate of 1 ml/min. All the solvents used were of HPLC grade. The phenolic compounds were identified by chromatographic comparison with authentic markers using standards including quercetin, gallic acid, vitamin C, vanillic acid, m-coumaric acid, sinapic acid, ferulic acid, caffeic acid, syringic acid, 4-OH-3 methoxy benzoic acid, trans-4-OH-3-methoxy cinamic acid, chlorogenic acid and chromatotropic acid. Phenolic compounds were quantified by peak area using authentic standards.

Biological activities

Brine shrimp lethality assay: Artificial sea water was prepared by mixing 3.8 g of sea salt/1000 ml of water. It was then filtered and poured in an unevenly divided large tank, eggs of the shrimp were then kept in bigger part of the tank and was later covered with aluminium foil. Eggs then hatched in the tank and larvae started to move in compartment. Temperature of the larvae's tank was adjusted at 37°C for the maturity and growth of the larvae. Concentration of 1000, 100, 10 micrograms per milliliters were prepared in the vials for testing and in three replicates from every dilution to make total of nine vials, to the 20 mg of sample. After that 2 ml of organic solvent was taken and from the solution 500, 50, 5 µl was used in same order. Solvents were allowed to evaporate under nitrogen by applying high vacuum for about thirty minutes. After the two days 10 shrimps were added to the 5 ml of sea water in each vial. These vials were then placed under light. After about on day number of survived shrimps were counted by the use of 3X magnifying glass. Data, thus obtained, was analyzed by probity analysis by the use of 95% confidence interval [16].

DPPH inhibition assay: A 10 µl of test solution was added in 96-wells plate followed by the addition of 90 µl of 100 µM methanol DPPH solution to make total volume up to 100 µl. The contents were mixed and incubated at 37°C for 30 min. The reduction in the absorbance was measured at 517 nm using Synergy HT BioTek® USA microplate reader. Quercetin was used as standard antioxidant. All experiments were carried out in triplicates. For the determination of IC50 values, test solutions were assayed at various dilutions i.e. 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.015 mM. Data obtained was computed on Ezi-fit software [17]. The decrease in absorbance indicates increased radical scavenging activity which was determined by the following formula.

$$\text{Inhibition(\%)} = \frac{(\text{Abs. of control} - \text{Abs. of test solution})}{\text{Abs. of control}} \times 100$$

Where: Absorbance of control=Total enzyme activity without inhibitor, Absorbance of test=Activity in the presence of test compound

Table 1. Results of extraction of *D. ramosa* with dichloromethane and methanol.

Plant name	Part used	Solvent used	Extract codes	Weight of extracts (gm)	% Yield (w/w)
<i>D. ramosa</i>	Whole plant	Dichloromethane	DRD	23 gm	15%
		Methanol	DRM	38 gm	25%

Table 2. Results of detection of secondary metabolites from the whole plants of *D. ramosa*.

Secondary metabolites	Alkaloids	Cardiac glycosides	Saponins	Tannins	Terpenoids	Flavonoids
Status	+	+	+	-	+	+

Absent: -; Present: +

Table 3. Total phenol, flavonoid and alkaloid content of dichloromethane and methanol extracts of *D. ramosa*.

Plant extract code	TPC	TFC	Alkaloid content
	(mg of *GA/g extract)	(mg of **RE/g extract)	(mg of ***AE/g extract)
DRM	199.2 ± 4.50	50.13 ± 3.51	23.92 ± 1.52
DRD	184.2 ± 4.04	73.02 ± 1.00	29.73 ± 4.04

Values are expressed as mean ± SEM of triplicate measurements.

*GA: Gallic acid; **RE: Rutin; ***AE: Atropine.

Results of identification and quantification of phenolic contents by HPLC showed the presence of chlorogenic acid, benzoic acid, ferulic acid, quercetin, syringic acid and vanillic acid in dichloromethane extract. Whereas caffeic acid, chlorogenic acid, cinnamic acid, gallic acid, p-coumaric acid, m-coumaric acid, quercetin, syringic acid and vanillic acid was present in methanol extract. Tables 4 and 5 show the retention time, area (mV.s), area (%) and concentration of the various

Results and Discussion

The shade dried whole plant of *D. ramosa* was carried for extraction process through the mechanism of maceration with dichloromethane and methanol sequentially. The extraction results, along with their assigned codes used are given in Table 1.

For the purpose of detection of alkaloids, tests were done by using Mayer's reagents, Wagner's reagents, Hager's reagents and Dragendorff's reagents. Keller kiliani test was done for detection of cardiac glycosides. The chemical test for detection of saponins was performed. The obtained results are given in Table 2.

Results of total phenolic, flavonoid and alkaloid content are summarized in Table 3. Methanol extract exhibited higher phenolic content as compared to the dichloromethane extract. While dichloromethane extract showed high flavonoid and alkaloid contents. Values are expressed as mean ± SEM of triplicate measurements.

phenolic compounds found in methanol and dichloromethane extracts respectively.

Brine shrimp (*Artemiasalina*) lethality bioassay was carried out against both dichloromethane and methanol extracts of *D. ramosa* for cytotoxic evaluation. Dichloromethane extract exhibited a very significant cytotoxic activity with "LD50

0.6903 µg/ml". No cytotoxic activity was showed by methanol extract. Results are given in Table 6.

Table 4. Identification and quantification of methanol extract of *D. ramosa*.

S.No.	Name of compounds	Retention time (min)	Area (mV.s)	Area (%)	Concentration (ppm)
1	Caffeic acid	12.2	39.313	4.3	1.81
2	Chlorogenic acid	15.42	8.344	0.9	0.64
3	Cinnamic acid	26.247	30.625	3.4	0.39
4	Gallic acid	4.867	17.769	2	0.63
5	m-coumaric acid	20.34	25.159	2.8	0.31
6	p-coumaric acid	17.32	7.619	0.8	0.09
7	Quercetin	3.213	36.456	4	1.92
8	Syringic acid	16.3	51.854	5.7	1.29
9	Vanillic acid	13.66	49.736	5.5	3.81

Table 5. Identification and quantification of dichloromethane extract of *D. ramosa*.

S.No.	Name of compounds	Retention time	Area (mV.s)	Area (%)	Concentration (ppm)
1	Chlorogenic acid	15.92	24.615	2.3	2.69
2	Benzoic acid	14.807	52.176	4.9	5.51
3	Ferulic acid	22.6	21.104	2	1.51s
4	Quercetin	3.127	29.818	2.8	1.57
5	Syringic acid	16.753	92.278	8.6	2.35
6	Vanillic acid	13.773	28.877	2.7	1.78

Table 6. Results of Brine shrimp (*Artemiasalina*) Lethality bioassay of dichloromethane and methanol extract of whole plant of *D. ramosa*.

Extract code	Dose (ug/ml)	No. of shrimps	No. of survivors	LD50 (ug/ml)	Standard drug	LD50 (ug/ml)
	1000	30	0			
	100	30	2			
DRD	10	30	6	0.6903	Etoposide	7.4625
	1000	30	22			
	100	30	28			
DRM	10	30	29	10246.36	Etoposide	7.4625

Table 7 represent the antioxidant activity of dichloromethane and methanol extracts of *D. ramosa*. Dichloromethane extract showed 55.37 ± 3.055 DPPH (%) inhibition \pm SEM while methanol extract showed 72.76 ± 1.52 DPPH (%) inhibition \pm SEM.

Herbal plants rich in phenolic compounds are progressively being utilized in food industry due to their retarding effect on oxidative degradation of lipids as well as can improve the nutritional value and quality of food [18].

Table 7. DPPH %inhibition of dichloromethane and methanol extract of *D. ramosa*.

Plant extract code	DPPH inhibition (%)
DRM	72.76 ± 1.52
DRD	55.37 ± 3.055
Quercetin (Standard)	74.54 ± 1.03

Values are expressed as mean \pm SEM of triplicate measurements.

Higher total phenolic content was found in the methanol extract 199.2 ± 4.50 (mg of GA/g of extract) than that of dichloromethane 184.2 ± 4.04 (mg of GA/g of extract).

Phenolic compounds of plants are categorized into various categories; major among these are flavonoids which have promising antioxidant activities [19]. Flavonoids are naturally present in plants and are known to produce positive effect on human health. Studies have proved that flavonoids have diverse range of antiviral, anti-inflammatory, antibacterial, anti-allergic and anticancer activities [20]. Moreover, flavonoids possess strong scavenging effect on oxidizing molecules like singlet oxygen and various free radicals [21] involved in various diseases. In our study higher flavonoid content was found in dichloromethane extract with 73.02 ± 1.00 (mg of extract) as compared to that of methanol extract having 50.13 ± 3.51 (mg of RE/g of extract) (Figures 1 and 2).

The electron donating capacity of herbal plants can be determined 2,20-diphenyl-1- picrylhydrazyl radical (DPPH), purple colored solution bleaching. The method is based on the principle that DPPH solution is scavenged and decolorized on the addition of some radical species or antioxidants. The change in color is directly proportional to the concentration of antioxidants. An enormous decrease is observed in the absorbance of reaction mixture which indicates the significant radical scavenging activity of the compound under test [20]. In the present study methanol extract showed higher free radical scavenging activity with $72.76 \pm 1.52\%$ inhibition of DPPH as compared to the dichloromethane extract showing $55.37 \pm 3.055\%$ inhibition of DPPH. Studies have proved that methanol extract may possess phenolic and hydroxyl-phenolic compounds with alcohol, sugar, acids and glycosides [22]. Higher antioxidant activity of methanol extract of *D. ramosa* may be due to the presence of these compounds or flavonoids.

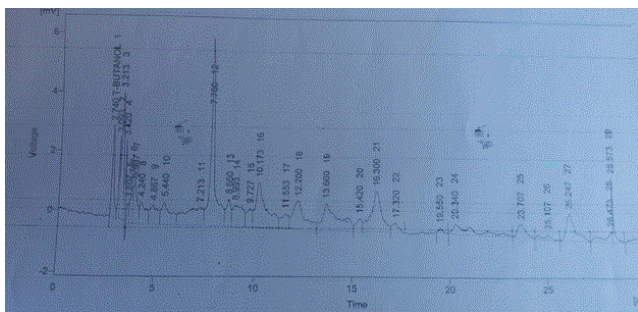


Figure 1. Identification and quantification of phenolic contents of methanol extract of *D. ramosa* by HPLC

In cytotoxic assay that is carried out through Brine Shrimp (*Artemiasalina*) Lethality Assay, the DCM extract show very high potential for Cytotoxicity. Results revealed that the LD50 of dichloromethane extract is $0.6903 \mu\text{l/ml}$ as standardized with Etoposide with LD50 of $7.4625 \mu\text{l/ml}$. It is concluded that dichloromethane extract of *D. ramosa* is 10 time more potent than Etoposide. Previous studies have proved that some flavonoids like quercetin possesses anticancer activity and inhibited the growth of cancer cells [23]. Gallic acid had free radical scavenging effect and induced apoptosis and differentiation in leukemia, lymphocyte cells, colon adenocarcinoma cell lines and lung cancer [24]. It has been proved that gallic acid have key role in the hindrance of cancer development and malignant transformation just like quercetin.

Thus it may be attributed that plant possesses high cytotoxic effect due to the presence of high flavonoid content in the plant.

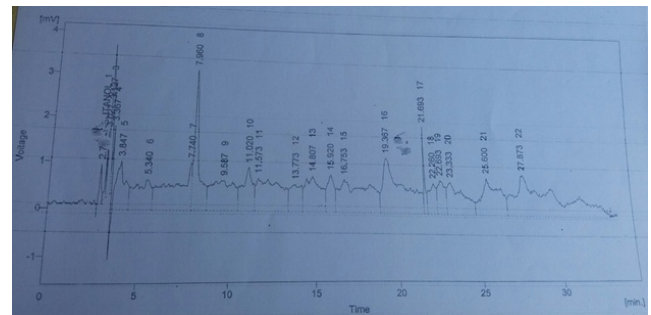


Figure 2. Identification and quantification of phenolic contents of dichloromethane extract of *D. ramosa* by HPLC.

Alkaloids have diverse range of biological properties involving antibacterial and anticancer activities and thus are responsible for the healing properties of natural medicine [25]. The present study showed the presence of significant level of alkaloids in both extracts. In dichloromethane extract alkaloid content was found to be 29.73 ± 4.04 (mg of AE/g of extract) whereas in methanol extract it was 23.92 ± 1.52 (mg of AE/g of extract).

Conclusion

The present study provides the evidence that the *D. ramosa* have prominent cytotoxic and antioxidant potential due to the presence of higher phenol, flavonoid and alkaloid content in the plant. Future research may be based on systemic investigation, fractionation, purification and isolation of antioxidants and cytotoxic agents for the development of novel, target oriented, more effective antioxidants and cytotoxic agents with safe therapeutic index. Further, work related to the isolation of the active constituents through bioassay-directed fractionation is in progress in our laboratory.

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Conflict of Interest

No conflict of interest associated with this study.

Contribution of Authors

It is declared that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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